Substitute Specification

Lawsonia intracellularis subunit vaccines.

The present invention relates i.a. to nucleic acids encoding novel *Lawsonia intracellularis* proteins, to DNA fragments, recombinant DNA molecules and live recombinant carriers comprising these sequences, to host cells comprising such nucleic acids, DNA fragments, recombinant DNA molecules and live recombinant carriers, to proteins encoded by these nucleotide sequences and to their use for the manufacturing of vaccines, to vaccines for combating *Lawsonia intracellularis* infections and methods for the preparation thereof and to diagnostic tests for the detection of *Lawsonia intracellularis* antigens and for the detection of antibodies against *Lawsonia intracellularis*.

Porcine proliferative enteropathy (PPE or PE) has become an important disease of the modern pig industry world-wide. The disease affects 15% to 50% of the growing herds and up to 30% of the individual animals in established problem herds. Today annual economical losses have been estimated US$ 5-10 in extra feed and facility time costs per affected pig. PPE is a group of chronic and acute conditions of widely differing clinical signs (death, pale and anaemic animals, watery, dark or bright red diarrhoea, depression, reduced appetite and reluctance to move, and retarded growth). However there are two consistent features. The first, a pathological change only visible at necropsy, is a thickening of the small intestine and colon mucosa. The second is the occurrence of intracytoplasmatic small-curved bacteria in the enterocytes of the affected intestine. These bacteria have now been established as the etiological agent of PPE and have been named *Lawsonia intracellularis*.

Over the years *Lawsonia intracellularis* has been found to affect a large group of animals including monkeys, rabbits, ferrets, hamsters, fox, horses, and other animals as diverse as ostrich and emu. *Lawsonia intracellularis* is a gram-negative, flagellated bacterium that multiplies in eukaryotic enterocytes only and no cell-free culture has been described. In order to persist and multiply in the cell *Lawsonia intracellularis*

must penetrate dividing crypt cells. The bacterium associates with the cell membrane and quickly enters the enterocyte via an entry vacuole. This then rapidly breaks down (within 3 hours) and the bacteria flourish and multiply freely in the cytoplasm. The mechanisms by which the bacteria cause infected cells to fail to mature, continue to undergo mitosis and form hypoplastic crypt cells is not yet understood.

The current understanding of *Lawsonia intracellularis* infection, treatment and control of the disease has been hampered by the fact that *Lawsonia intracellularis* can not be cultivated in cell-free media. Although there are reports of successful co-culturing *Lawsonia intracellularis* in rat enterocytes this has not led to the development of inactivated vaccines for combating *Lawsonia intracellularis*, although there clearly is a need for such vaccines.

It is an objective of the present invention to provide a vaccine for combating *Lawsonia intracellularis* infection.

It was surprisingly found now, that *Lawsonia intracellularis* produces nine novel proteins each of which is capable, separately or in combination with any of the other of these nine novel proteins, to induce protective immunity against *Lawsonia intracellularis*.

The first of these nine novel proteins will be referred to as the 75 kD protein. The amino acid sequence of the novel protein is presented in sequence identifier SEQ ID NO: 2. The gene encoding this protein has been sequenced and its nucleic acid sequence is shown in sequence identifier SEQ ID NO: 1. The gene will also be referred to in the Examples as "gene 5074".

The second of these nine novel proteins will be referred to as the 27kD protein. The amino acid sequence of the novel protein is presented in sequence identifier SEQ ID NO: 4. The gene encoding this protein has been sequenced and its nucleic acid sequence is shown in sequence identifier SEQ ID NO: 3. The gene will also be referred to in the Examples as "gene 5669".

The third of these nine novel proteins will be referred to as the 62 kD protein. The amino acid sequence of the novel protein is presented in sequence identifier SEQ ID NO: 6. The gene encoding this protein has been sequenced and its nucleic acid sequence is shown in sequence identifier SEQ ID NO: 5. The gene will also be referred to in the Examples as "gene 4423".

The fourth of these nine novel proteins will be referred to as the 57 kD protein. The amino acid sequence of the novel protein is presented in sequence identifier SEQ ID NO: 8. The gene encoding this protein has been sequenced and its nucleic acid sequence is shown in sequence identifier SEQ ID NO: 7. The gene will also be referred to in the Examples as "gene 3123".

The fifth of these nine novel proteins will be referred to as the 74 kD protein. The amino acid sequence of the novel protein is presented in sequence identifier SEQ ID NO: 10. The gene encoding this protein has been sequenced and its nucleic acid sequence is shown in sequence identifier SEQ ID NO: 9. The gene will also be referred to in the Examples as "gene 5293".

The sixth of these nine novel proteins will be referred to as the 44 kD protein. The amino acid sequence of the novel protein is presented in sequence identifier SEQ ID NO: 12. The gene encoding this protein has been sequenced and its nucleic acid sequence is shown in sequence identifier SEQ ID NO: 11. The gene will also be referred to in the Examples as "gene 5464".

The seventh of these nine novel proteins will be referred to as the 43 kD protein. The amino acid sequence of the novel protein is presented in sequence identifier SEQ ID NO: 14. The gene encoding this protein has been sequenced and its nucleic acid sequence is shown in sequence identifier SEQ ID NO: 13. The gene will also be referred to in the Examples as "gene 5473".

The eighth of these nine novel proteins will be referred to as the 26/31 kD protein.

The amino acid sequence of the novel protein is presented in sequence identifier SEQ ID NO: 16. The gene encoding this protein has been sequenced and its nucleic acid sequence is shown in sequence identifier SEQ ID NO: 15. The gene will also be referred to in the Examples as "gene 4320".

The ninth of these nine novel proteins will be referred to as the 101 kD protein.
The amino acid sequence of the novel protein is presented in sequence identifier SEQ ID NO: 18. The gene encoding this protein has been sequenced and its nucleic acid sequence is shown in sequence identifier SEQ ID NO: 17. The gene will also be referred to in the Examples as "gene 2008".

It is well-known in the art, that many different nucleic acid sequences can encode one and the same protein. This phenomenon is commonly known as wobble in the second and especially the third base of each triplet encoding an amino acid. This phenomenon can result in a heterology of about 30% for two nucleic acid sequences still encoding the same protein. Therefore, two nucleic acid sequences having a sequence homology of about 70 % can still encode one and the same protein.

Thus, one embodiment relates to nucleic acids encoding a *Lawsonia intracellularis* protein and to parts of that nucleic acid that encode an immunogenic fragment of that protein, wherein those nucleic acids or parts thereof have a level of homology with the nucleic acid of which the sequence is given in SEQ ID NO: 1 of at least 90 %.

Preferably, the nucleic acid encoding this *Lawsonia intracellularis* protein or the part of said nucleic acid has at least 92 %, preferably 94 %, more preferably 95 % and even more preferably 96% homology with the nucleic acid having the sequence given in SEQ ID NO: 1. Even more preferred is a homology level of 98 % or even 100 %.

The level of nucleotide homology can be determined with the computer program "BLAST 2 SEQUENCES" by selecting sub-program: "BLASTN."
A reference for this program is Tatiana A. Tatusova, Thomas L. Madden FEMS Microbiol. Letters 174: 247-250 (1999). Parameters used are the default parameters:

Reward for a match: +1. Penalty for a mismatch: -2. Open gap: 5. Extension gap: 2. Gap x_dropoff: 50.

Another approach for deciding if a certain nucleic acid is or is not a nucleic acid according to the invention relates to the question if that certain nucleic acid does hybridize under stringent conditions to nucleic acids having the nucleotide sequence as depicted in SEQ ID NO: 1.
If a nucleic acid hybridizes under stringent conditions to the nucleotide sequence as depicted in SEQ ID NO: 1, it is considered to be a nucleic acid according to the invention.

The definition of stringent conditions follows from the formula of Meinkoth and Wahl (1984. Hybridization of nucleic acids immobilized on solid supports. Anal. Biochem. 138: 267-284.)

Tm = [81.5°C + 16.6(log M) + 0.41(%GC) - 0.61(%formamide) - 500/L] - 1°C/1%mismatch

In this formula, M is molarity of monovalent cations; %GC is the percentage of guanosine and cytosine nucleotides in the DNA; L is the length of the hybrid in base pairs.

Stringent conditions are those conditions under which nucleic acids or fragments thereof still hybridize, if they have a mismatch of 10% at the most, to the nucleic acid having the sequence depicted in SEQ ID NO: 1.

A second embodiment relates to nucleic acids encoding a *Lawsonia intracellularis* protein and to parts of that nucleic acid that encode an immunogenic fragment of that protein, wherein those nucleic acids or parts thereof have a level of homology with the nucleic acid of which the sequence is given in SEQ ID NO: 3 of at least 90 %.

Preferably, the nucleic acid encoding this *Lawsonia intracellularis* protein or the part of said nucleic acid has at least 92 %, preferably 94 %, more preferably 95 % and

even more preferably 96% homology with the nucleic acid having the sequence given in SEQ ID NO: 3. Even more preferred is a homology level of 98 % or even 100 %.

A third embodiment relates to nucleic acids encoding a *Lawsonia intracellularis* protein and to parts of that nucleic acid that encode an immunogenic fragment of that protein, wherein those nucleic acids or parts thereof have a level of homology with the nucleic acid of which the sequence is given in SEQ ID NO: 5 of at least 90 %.

Preferably, the nucleic acid encoding this *Lawsonia intracellularis* protein or the part of said nucleic acid has at least 92 %, preferably 94 %, more preferably 95 % and even more preferably 96% homology with the nucleic acid having the sequence given in SEQ ID NO: 5. Even more preferred is a homology level of 98 % or even 100 %.

A fourth embodiment relates to nucleic acids encoding a *Lawsonia intracellularis* protein and to parts of that nucleic acid that encode an immunogenic fragment of that protein, wherein those nucleic acids or parts thereof have a level of homology with the nucleic acid of which the sequence is given in SEQ ID NO: 7 of at least 90 %.

Preferably, the nucleic acid encoding this *Lawsonia intracellularis* protein or the part of said nucleic acid has at least 92 %, preferably 94 %, more preferably 95 % and even more preferably 96% homology with the nucleic acid having the sequence given in SEQ ID NO: 7. Even more preferred is a homology level of 98 % or even 100 %.

A fifth embodiment relates to nucleic acids encoding a *Lawsonia intracellularis* protein and to parts of that nucleic acid that encode an immunogenic fragment of that protein, wherein those nucleic acids or parts thereof have a level of homology with the nucleic acid of which the sequence is given in SEQ ID NO: 9 of at least 90 %.

Preferably, the nucleic acid encoding this *Lawsonia intracellularis* protein or the part of said nucleic acid has at least 92 %, preferably 94 %, more preferably 95 % and even more preferably 96% homology with the nucleic acid having the sequence given in SEQ ID NO: 9. Even more preferred is a homology level of 98 % or even 100 %.

A sixth embodiment relates to nucleic acids encoding a *Lawsonia intracellularis* protein and to parts of that nucleic acid that encode an immunogenic fragment of that protein, wherein those nucleic acids or parts thereof have a level of homology with the nucleic acid of which the sequence is given in SEQ ID NO: 11 of at least 90 %.

Preferably, the nucleic acid encoding this *Lawsonia intracellularis* protein or the part of said nucleic acid has at least 92 %, preferably 94 %, more preferably 95 % and even more preferably 96% homology with the nucleic acid having the sequence given in SEQ ID NO: 11. Even more preferred is a homology level of 98 % or even 100 %.

A seventh embodiment relates to nucleic acids encoding a *Lawsonia intracellularis* protein and to parts of that nucleic acid that encode an immunogenic fragment of that protein, wherein those nucleic acids or parts thereof have a level of homology with the nucleic acid of which the sequence is given in SEQ ID NO: 13 of at least 90 %.

Preferably, the nucleic acid encoding this *Lawsonia intracellularis* protein or the part of said nucleic acid has at least 92 %, preferably 94 %, more preferably 95 % and even more preferably 96% homology with the nucleic acid having the sequence given in SEQ ID NO: 13. Even more preferred is a homology level of 98 % or even 100 %.

An eighth embodiment relates to nucleic acids encoding a *Lawsonia intracellularis* protein and to parts of that nucleic acid that encode an immunogenic fragment of that protein, wherein those nucleic acids or parts thereof have a level of homology with the nucleic acid of which the sequence is given in SEQ ID NO: 15 of at least 90 %.

Preferably, the nucleic acid encoding this *Lawsonia intracellularis* protein or the part of said nucleic acid has at least 92 %, preferably 94 %, more preferably 95 % and even more preferably 96% homology with the nucleic acid having the sequence given in SEQ ID NO: 15. Even more preferred is a homology level of 98 % or even 100 %.

A ninth embodiment relates to nucleic acids encoding a *Lawsonia intracellularis* protein and to parts of that nucleic acid that encode an immunogenic fragment of that protein, wherein those nucleic acids or parts thereof have a level of homology with the nucleic acid of which the sequence is given in SEQ ID NO: 17 of at least 90 %.

Preferably, the nucleic acid encoding this *Lawsonia intracellularis* protein or the part of said nucleic acid has at least 92 %, preferably 94 %, more preferably 95 % and even more preferably 96% homology with the nucleic acid having the sequence given in SEQ ID NO: 17. Even more preferred is a homology level of 98 % or even 100 %.

Since the present invention discloses nucleic acids encoding novel *Lawsonia intracellularis* proteins, it is now for the first time possible to obtain these proteins in sufficient quantities. This can e.g. be done by using expression systems to express the genes encoding the proteins.
Therefore, in a more preferred embodiment, the invention relates to DNA fragments comprising a nucleic acid according to the invention. Such DNA fragments can e.g. be plasmids, into which a nucleic acid according to the invention is cloned. Such DNA fragments are e.g. useful for enhancing the amount of DNA for use as a primer, as described below.

An essential requirement for the expression of the nucleic acid is an adequate promoter functionally linked to the nucleic acid, so that the nucleic acid is under the control of the promoter. It is obvious to those skilled in the art that the choice of a promoter extends to any eukaryotic, prokaryotic or viral promoter capable of directing gene transcription in cells used as host cells for protein expression.
Therefore, an even more preferred form of this embodiment relates to a recombinant DNA molecule comprising a DNA fragment or a nucleic acid according to the invention that is placed under the control of a functionally linked promoter. This can be accomplished by means of e.g. standard molecular biology techniques. (Sambrook, J. and Russell, D.W., Molecular cloning: a laboratory manual, 2001. ISBN 0-87969-577-3).

Functionally linked promoters are promoters that are capable of controlling the transcription of the nucleic acids to which they are linked.
Such a promoter can be a Lawsonia promoter e.g. the promoter involved in *in vivo* expression of any of the genes encoding the nine novel proteins, provided that that promoter is functional in the cell used for expression. It can also be a heterologous promoter. When the host cells are bacteria, useful expression control sequences which may be used include the Trp promoter and operator (Goeddel, et al., Nucl. Acids Res., 8, 4057, 1980); the lac promoter and operator (Chang, et al., Nature, 275, 615, 1978); the outer membrane protein promoter (Nakamura, K. and Inouge, M., EMBO J., 1, 771-775, 1982); the bacteriophage lambda promoters and operators (Remaut, E. et al., Nucl. Acids Res., 11, 4677-4688, 1983); the α-amylase (B. subtilis) promoter and operator, termination sequences and other expression enhancement and control sequences compatible with the selected host cell.
When the host cell is yeast, useful expression control sequences include, e.g., α-mating factor. For insect cells the polyhedrin or p10 promoters of baculoviruses can be used (Smith, G.E. et al., Mol. Cell. Biol. 3, 2156-65, 1983). When the host cell is of mammalian origin illustrative useful expression control sequences include the SV-40 promoter (Berman, P.W. et al., Science, 222, 524-527, 1983) or the metallothionein promoter (Brinster, R.L., Nature, 296, 39-42, 1982) or a heat shock promoter (Voellmy et al., Proc. Natl. Acad. Sci. USA, 82, 4949-53, 1985).

Bacterial, yeast, fungal, insect and mammalian cell expression systems are very frequently used systems. Such systems are well-known in the art and generally available, e.g. commercially through Invitrogen, Novagen or Clontech Laboratories, Inc. 4030 Fabian Way, Palo Alto, California 94303-4607, USA. Next to these expression systems, parasite-based expression systems are very attractive expression systems. Such systems are e.g. described in the French Patent Application with Publication number 2 714 074, and in US NTIS Publication No US 08/043109 (Hoffman, S. and Rogers, W.: Public. Date 1 December 1993).

A still even more preferred form of this embodiment of the invention relates to Live Recombinant Carriers (LRCs) comprising a nucleic acid encoding any of the genes

encoding the nine novel proteins or an immunogenic fragment thereof according to the invention, a DNA fragment according to the invention or a recombinant DNA molecule according to the invention. Such carriers are e.g. bacteria and viruses. These LRCs are micro-organisms or viruses in which additional genetic information, in this case a nucleic acid encoding any of the genes encoding the nine novel proteins or an immunogenic fragment thereof according to the invention has been cloned. Animals infected with such LRCs will produce an immunogenic response not only against the immunogens of the carrier, but also against the immunogenic parts of the protein(s) for which the genetic code is additionally cloned into the LRC, e.g. the 75 kD protein or any of the other proteins according to the invention.
As an example of bacterial LRCs, attenuated Salmonella strains known in the art can attractively be used.
Live recombinant carrier parasites have i.a. been described by Vermeulen, A. N. (Int. Journ. Parasitol. 28: 1121-1130 (1998))
Also, LRC viruses may be used as a way of transporting the nucleic acid into a target cell. Live recombinant carrier viruses are also called vector viruses. Viruses often used as vectors are Vaccinia viruses (Panicali et al; Proc. Natl. Acad. Sci. USA, 79: 4927 (1982), Herpesviruses (E.P.A. 0473210A2), and Retroviruses (Valerio, D. et al; in Baum, S.J., Dicke, K.A., Lotzova, E. and Pluznik, D.H. (Eds.), Experimental Haematology today - 1988. Springer Verlag, New York: pp. 92-99 (1989)).

The technique of *in vivo* homologous recombination, well-known in the art, can be used to introduce a recombinant nucleic acid into the genome of a bacterium, parasite or virus of choice, capable of inducing expression of the inserted nucleic acid according to the invention in the host animal.

Finally another form of this embodiment of the invention relates to a host cell comprising a nucleic acid encoding a protein according to the invention, a DNA fragment comprising such a nucleic acid or a recombinant DNA molecule comprising such a nucleic acid under the control of a functionally linked promoter. This form also relates to a host cell containing a live recombinant carrier containing a nucleic acid

molecule encoding any of the genes encoding the nine novel proteins or a fragment thereof according to the invention.
A host cell may be a cell of bacterial origin, e.g. *Escherichia coli*, *Bacillus subtilis* and *Lactobacillus* species, in combination with bacteria-based plasmids as pBR322, or bacterial expression vectors as pGEX, or with bacteriophages. The host cell may also be of eukaryotic origin, e.g. yeast-cells in combination with yeast-specific vector molecules, or higher eukaryotic cells like insect cells (Luckow et al; Bio-technology 6: 47-55 (1988)) in combination with vectors or recombinant baculoviruses, plant cells in combination with e.g. Ti-plasmid based vectors or plant viral vectors (Barton, K.A. et al; Cell 32: 1033 (1983), mammalian cells like Hela cells, Chinese Hamster Ovary cells (CHO) or Crandell Feline Kidney-cells, also with appropriate vectors or recombinant viruses.

Another embodiment of the invention relates to the novel proteins and to immunogenic fragments thereof according to the invention.

The concept of immunogenic fragments will be defined below.

One form of this embodiment relates i.a. to *Lawsonia intracellularis* proteins that have an amino acid sequence that is at least 90 % homologous to the amino acid sequence as depicted in SEQ ID NO: 2 and to immunogenic fragments of said protein.

In a preferred form, the embodiment relates to such *Lawsonia intracellularis* proteins that have a sequence homology of at least 92 %, preferably 94 %, more preferably 96 % homology to the amino acid sequence as depicted in SEQ ID NO: 2 and to immunogenic fragments of such proteins.
Even more preferred is a homology level of 98 % or even 100 %.

The level of protein homology can be determined with the computer program "BLAST 2 SEQUENCES" by selecting sub-program: "BLASTP."

A reference for this program is Tatiana A. Tatusova, Thomas L. Madden FEMS Microbiol. Letters 174: 247-250 (1999). Matrix used: "blosum62". Parameters used are the default parameters:
Open gap: 11. Extension gap: 1. Gap x_dropoff: 50.

It will be understood that, for the particular proteins embraced herein, natural variations can exist between individual *Lawsonia intracellularis* strains. These variations may be demonstrated by (an) amino acid difference(s) in the overall sequence or by deletions, substitutions, insertions, inversions or additions of (an) amino acid(s) in said sequence. Amino acid substitutions which do not essentially alter biological and immunological activities, have been described, e.g. by Neurath et al in "The Proteins" Academic Press New York (1979). Amino acid replacements between related amino acids or replacements which have occurred frequently in evolution are, inter alia, Ser/Ala, Ser/Gly, Asp/Gly, Asp/Asn, Ile/Val (see Dayhof, M.D., Atlas of protein sequence and structure, Nat. Biomed. Res. Found., Washington D.C., 1978, vol. 5, suppl. 3). Other amino acid substitutions include Asp/Glu, Thr/Ser, Ala/Gly, Ala/Thr, Ser/Asn, Ala/Val, Thr/Phe, Ala/Pro, Lys/Arg, Leu/Ile, Leu/Val and Ala/Glu. Based on this information, Lipman and Pearson developed a method for rapid and sensitive protein comparison (Science,227, 1435-1441, 1985) and determining the functional similarity between homologous proteins. Such amino acid substitutions of the exemplary embodiments of this invention, as well as variations having deletions and/or insertions are within the scope of the invention as long as the resulting proteins retain their immune reactivity.
This explains why *Lawsonia intracellularis* proteins according to the invention, when isolated from different field isolates, may have homology levels of about 90 %, while still representing the same protein with the same immunological characteristics.
Those variations in the amino acid sequence of a certain protein according to the invention that still provide a protein capable of inducing an immune response against infection with *Lawsonia intracellularis* or at least against the clinical manifestations of the infection are considered as “not essentially influencing the immunogenicity”.

A second form of this embodiment relates i.a. to *Lawsonia intracellularis* proteins that have an amino acid sequence that is at least 90 % homologous to the amino acid sequence as depicted in SEQ ID NO: 4 and to immunogenic fragments of said protein.

In a preferred form, the embodiment relates to such *Lawsonia intracellularis* proteins that have a sequence homology of at least 92 %, preferably 94 %, more preferably 96 % homology to the amino acid sequence as depicted in SEQ ID NO: 4 and to immunogenic fragments of such proteins.
Even more preferred is a homology level of 98 % or even 100 %.

A third form of this embodiment relates i.a. to *Lawsonia intracellularis* proteins that have an amino acid sequence that is at least 90 % homologous to the amino acid sequence as depicted in SEQ ID NO: 6 and to immunogenic fragments of said protein.

In a preferred form, the embodiment relates to such *Lawsonia intracellularis* proteins that have a sequence homology of at least 92 %, preferably 94 %, more preferably 96 % homology to the amino acid sequence as depicted in SEQ ID NO: 6 and to immunogenic fragments of such proteins.
Even more preferred is a homology level of 98 % or even 100 %.

A fourth form of this embodiment relates i.a. to *Lawsonia intracellularis* proteins that have an amino acid sequence that is at least 90 % homologous to the amino acid sequence as depicted in SEQ ID NO: 8 and to immunogenic fragments of said protein.

In a preferred form, the embodiment relates to such *Lawsonia intracellularis* proteins that have a sequence homology of at least 92 %, preferably 94 %, more preferably 96 % homology to the amino acid sequence as depicted in SEQ ID NO: 8 and to immunogenic fragments of such proteins.
Even more preferred is a homology level of 98 % or even 100 %.

A fifth form of this embodiment relates i.a. to *Lawsonia intracellularis* proteins that have an amino acid sequence that is at least 90 % homologous to the amino acid

sequence as depicted in SEQ ID NO: 10 and to immunogenic fragments of said protein.

In a preferred form, the embodiment relates to such *Lawsonia intracellularis* proteins that have a sequence homology of at least 92 %, preferably 94 %, more preferably 96 % homology to the amino acid sequence as depicted in SEQ ID NO: 10 and to immunogenic fragments of such proteins.
Even more preferred is a homology level of 98 % or even 100 %.

A sixth form of this embodiment relates i.a. to *Lawsonia intracellularis* proteins that have an amino acid sequence that is at least 90 % homologous to the amino acid sequence as depicted in SEQ ID NO: 12 and to immunogenic fragments of said protein.

In a preferred form, the embodiment relates to such *Lawsonia intracellularis* proteins that have a sequence homology of at least 92 %, preferably 94 %, more preferably 96 % homology to the amino acid sequence as depicted in SEQ ID NO: 12 and to immunogenic fragments of such proteins.
Even more preferred is a homology level of 98 % or even 100 %.

A seventh form of this embodiment relates i.a. to *Lawsonia intracellularis* proteins that have an amino acid sequence that is at least 90 % homologous to the amino acid sequence as depicted in SEQ ID NO: 14 and to immunogenic fragments of said protein.

In a preferred form, the embodiment relates to such *Lawsonia intracellularis* proteins that have a sequence homology of at least 92 %, preferably 94 %, more preferably 96 % homology to the amino acid sequence as depicted in SEQ ID NO: 14 and to immunogenic fragments of such proteins.
Even more preferred is a homology level of 98 % or even 100 %.

An eighth form of this embodiment relates i.a. to *Lawsonia intracellularis* proteins that have an amino acid sequence that is at least 90 % homologous to the amino acid sequence as depicted in SEQ ID NO: 16 and to immunogenic fragments of said protein.

In a preferred form, the embodiment relates to such *Lawsonia intracellularis* proteins that have a sequence homology of at least 92 %, preferably 94 %, more preferably 96 % homology to the amino acid sequence as depicted in SEQ ID NO: 16 and to immunogenic fragments of such proteins.
Even more preferred is a homology level of 98 % or even 100 %.

A ninth form of this embodiment relates i.a. to *Lawsonia intracellularis* proteins that have an amino acid sequence that is at least 90 % homologous to the amino acid sequence as depicted in SEQ ID NO: 18 and to immunogenic fragments of said protein.

In a preferred form, the embodiment relates to such *Lawsonia intracellularis* proteins that have a sequence homology of at least 92 %, preferably 94 %, more preferably 96 % homology to the amino acid sequence as depicted in SEQ ID NO: 18 and to immunogenic fragments of such proteins.
Even more preferred is a homology level of 98 % or even 100 %.

When a protein is used for e.g. vaccination purposes or for raising antibodies, it is however not necessary to use the whole protein. It is also possible to use a fragment of that protein that is capable, as such or coupled to a carrier such as e.g. KLH, of inducing an immune response against that protein, a so-called immunogenic fragment. An "immunogenic fragment" is understood to be a fragment of the full-length protein that still has retained its capability to induce an immune response in the host, i.e. comprises a B- or T-cell epitope. At this moment, a variety of techniques is available to easily identify DNA fragments encoding antigenic fragments (determinants). The method described by Geysen et al (Patent Application WO 84/03564, Patent Application WO 86/06487, US Patent NR. 4,833,092, Proc. Natl Acad. Sci. 81: 3998-

4002 (1984), J. Imm. Meth. 102, 259-274 (1987), the so-called PEPSCAN method is an easy to perform, quick and well-established method for the detection of epitopes; the immunologically important regions of the protein. The method is used world-wide and as such well-known to one skilled in the art. This (empirical) method is especially suitable for the detection of B-cell epitopes. Also, given the sequence of the gene encoding any protein, computer algorithms are able to designate specific protein fragments as the immunologically important epitopes on the basis of their sequential and/or structural agreement with epitopes that are now known. The determination of these regions is based on a combination of the hydrophilicity criteria according to Hopp and Woods (Proc. Natl. Acad. Sci. 78: 38248-3828 (1981)), and the secondary structure aspects according to Chou and Fasman (Advances in Enzymology 47: 45-148 (1987) and US Patent 4,554,101). T-cell epitopes can likewise be predicted from the sequence by computer with the aid of Berzofsky's amphiphilicity criterion (Science 235, 1059-1062 (1987) and US Patent application NTIS US 07/005,885). A condensed overview is found in: Shan Lu on common principles: Tibtech 9: 238-242 (1991), Good et al on Malaria epitopes; Science 235: 1059-1062 (1987), Lu for a review; Vaccine 10: 3-7 (1992), Berzowsky for HIV-epitopes; The FASEB Journal 5:2412-2418 (1991).

Therefore, one form of still another embodiment of the invention relates to vaccines capable of protecting pigs against *Lawsonia intracellularis* infection, that comprise a protein or an immunogenic fragment thereof, according to the invention as described above together with a pharmaceutically acceptable carrier.

Still another embodiment of the present invention relates to the proteins according to the invention for use in a vaccine.

Still another embodiment relates to the use of a protein according to the invention for the manufacturing of a vaccine for combating *Lawsonia intracellularis* infections.

One way of making a vaccine according to the invention is by biochemical purification of the proteins or immunogenic fragments thereof according to the

invention from bacteria obtained through mucosal scrapings taken from the infected intestine wall. This is however a very time-consuming way of making the vaccine.

It is therefore much more convenient to use the expression products of the genes encoding the proteins or immunogenic fragments thereof according to the invention in vaccines. The nucleic acid sequences of the genes encoding the nine novel proteins are provided by the present invention.

Such vaccines based upon the expression products of these genes can easily be made by admixing a protein according to the invention or an immunogenic fragment thereof according to the invention with a pharmaceutically acceptable carrier as described below.

Alternatively, a vaccine according to the invention can comprise live recombinant carriers as described above, capable of expressing the proteins according to the invention or immunogenic fragments thereof according to the invention. Such vaccines, e.g. based upon a Salmonella carrier or a viral carrier infecting the enteric epithelium, or e.g. the respiratory epithelium have the advantage over subunit vaccines that they better mimic the natural way of infection of *Lawsonia intracellularis*. Moreover, their self-propagation is an advantage since only low amounts of the recombinant carrier are necessary for immunization.

Vaccines described above all contribute to active vaccination, i.e. the host's immune system is triggered by a protein according to the invention or an immunogenic fragment thereof, to make antibodies against these proteins.
Alternatively, such antibodies can be raised in e.g. rabbits or can be obtained from antibody-producing cell lines as described below. Such antibodies can then be administered to the host animal. This method of vaccination, passive vaccination, is the vaccination of choice when an animal is already infected, and there is no time to allow the natural immune response to be triggered. It is also the preferred method for vaccinating immune-compromised animals. Administered antibodies against

Lawsonia intracellularis can in these cases bind directly to the bacteria. This has the advantage that it immediately decreases or stops *Lawsonia intracellularis* growth. Therefore, one other form of this embodiment of the invention relates to vaccines comprising antibodies against at least one of the novel *Lawsonia intracellularis* proteins according to the invention.

Vaccines can also be based upon host cells as described above, that comprise the proteins or immunogenic fragments thereof according to the invention.

An alternative and efficient way of vaccination is direct vaccination with DNA encoding the relevant antigen. Direct vaccination with DNA encoding proteins has been successful for many different proteins. (As reviewed in e.g. Donnelly et al., The Immunologist 2: 20-26 (1993)).
This way of vaccination is very attractive for the vaccination of pigs against *Lawsonia intracellularis* infection.
Therefore, still other forms of this embodiment of the invention relate to vaccines comprising nucleic acids encoding a protein according to the invention or immunogenic fragments thereof according to the invention, and to vaccines comprising DNA fragments that comprise such nucleic acids.
Still other forms of this embodiment relate to vaccines comprising recombinant DNA molecules according to the invention.
DNA vaccines can easily be administered through intradermal application e.g. using a needle-less injector. This way of administration delivers the DNA directly into the cells of the animal to be vaccinated. Amounts of DNA in the microgram range between 1 and 100 μg provide very good results.

In a further embodiment, the vaccine according to the present invention additionally comprises one or more antigens derived from other pig pathogenic organisms and viruses, or genetic information encoding such antigens.
Such organisms and viruses are preferably selected from the group of Pseudorabies virus, Porcine influenza virus, Porcine parvo virus, Transmissible gastro-enteritis virus, Rotavirus, *Escherichia coli, Erysipelothrix rhusiopathiae, Bordetella*

bronchiseptica, Salmonella cholerasuis, Haemophilus parasuis, Pasteurella multocida, Streptococcus suis, Mycoplasma hyopneumoniae, Brachyspira hyodysenteriae and *Actinobacillus pleuropneumoniae.*

All vaccines according to the present invention comprise a pharmaceutically acceptable carrier. A pharmaceutically acceptable carrier can be e.g. sterile water or a sterile physiological salt solution. In a more complex form the carrier can e.g. be a buffer.

Methods for the preparation of a vaccine comprise the admixing of a protein according to the invention, or an immunogenic fragment thereof, and a pharmaceutically acceptable carrier.

Vaccines according to the present invention may in a preferred presentation also contain an adjuvant. Adjuvants in general comprise substances that boost the immune response of the host in a non-specific manner. A number of different adjuvants are known in the art. Examples of adjuvants are Freunds Complete and Incomplete adjuvant, vitamin E, non-ionic block polymers, muramyldipeptides, Quill A(R), mineral oil e.g. Bayol(R) or Markol(R), vegetable oil, and Carbopol(R) (a homopolymer), or Diluvac(R) Forte.
The vaccine may also comprise a so-called "vehicle". A vehicle is a compound to which the polypeptide adheres, without being covalently bound to it. Often used vehicle compounds are e.g. aluminium hydroxide, -phosphate or -oxide, silica, Kaolin, and Bentonite.
A special form of such a vehicle, in which the antigen is partially embedded in the vehicle, is the so-called ISCOM (EP 109.942, EP 180.564, EP 242.380)
In addition, the vaccine may comprise one or more suitable surface-active compounds or emulsifiers, e.g., SPAN or TWEEN. Often, the vaccine is mixed with stabilizers, e.g. to protect degradation-prone polypeptides from being degraded, to enhance the shelf-life of the vaccine, or to improve freeze-drying efficiency. Useful stabilizers are i.a. SPGA (Bovarnik et al; J. Bacteriology 59: 509 (1950)), carbohydrates e.g. sorbitol, mannitol, trehalose, starch, sucrose, dextran or glucose, proteins such as

albumin or casein or degradation products thereof, and buffers, such as alkali metal phosphates.
In addition, the vaccine may be suspended in a physiologically acceptable diluent.
It goes without saying, that other ways of adjuvanating adding vehicle compounds or diluents, emulsifying or stabilizing a polypeptide are also embodied in the present invention.

Vaccines according to the invention can very suitably be administered in amounts ranging between 1 and 100 micrograms, although smaller doses can in principle be used. A dose exceeding 100 micrograms will, although immunologically very suitable, be less attractive for commercial reasons.

Vaccines based upon live attenuated recombinant carriers, such as the LRC-viruses and bacteria described above can be administered in much lower doses, because they multiply themselves during the infection. Therefore, very suitable amounts would range between 10^3 and 10^9 CFU/PFU for respectively bacteria and viruses.

Many ways of administration can be applied. Oral application is a very attractive way of administration, because the infection is an infection of the digestive tract. A preferred way of oral administration is the packaging of the vaccine in capsules, known and frequently used in the art, that only disintegrate after they have passed the highly acidic environment of the stomach. Also, the vaccine could be mixed with compounds known in the art for temporarily enhancing the pH of the stomach.
Systemic application is also suitable, e.g. by intramuscular application of the vaccine. If this route is followed, standard procedures known in the art for systemic application are well-suited.

From a point of view of protection against disease, a quick and correct diagnosis of *Lawsonia intracellularis* infection is important.
Therefore it is another objective of this invention to provide diagnostic tools suitable for the detection of *Lawsonia intracellularis* infection.

A diagnostic test for the detection of *Lawsonia intracellularis* antibodies in sera can be e.g. a simple standard sandwich-ELISA-test in which any of the novel proteins according to the invention or antigenic fragments thereof according to the invention are coated to the wall of the wells of an ELISA-plate. A method for the detection of such antibodies is e.g. incubation of the 75 kD protein (or any other protein according to the invention) or antigenic fragments thereof with serum from mammals to be tested, followed by e.g. incubation with a labelled antibody against the relevant mammalian antibody. A colour reaction can then reveal the presence or absence of antibodies against *Lawsonia intracellularis*. Another example of a diagnostic test system is e.g. the incubation of a Western blot comprising the 75 kD protein or an antigenic fragment thereof according to the invention, with serum of mammals to be tested, followed by analysis of the blot.

Thus, another embodiment of the present invention relates to diagnostic tests for the detection of antibodies against *Lawsonia intracellularis*. Such tests comprise a protein or a fragment thereof according to the invention.

A diagnostic test based upon the detection of antigenic material of any of the nine proteins of *Lawsonia intracellularis* antigens and therefore suitable for the detection of *Lawsonia intracellularis* infection can e.g. also be a standard ELISA test. In one example of such a test the walls of the wells of an ELISA plate are coated with antibodies directed against the 75 kD protein (or any other protein according to the invention). After incubation with the material to be tested, labelled anti-*Lawsonia intracellularis* antibodies are added to the wells. A colour reaction then reveals the presence of antigenic material from *Lawsonia intracellularis*.
Therefore, still another embodiment of the present invention relates to diagnostic tests for the detection of antigenic material of *Lawsonia intracellularis*. Such tests comprise antibodies against a protein or a fragment thereof according to the invention.

The polypeptides or immunogenic fragments thereof according to the invention expressed as characterized above can be used to produce antibodies, which may be polyclonal, monospecific or monoclonal (or derivatives thereof). If polyclonal

antibodies are desired, techniques for producing and processing polyclonal sera are well-known in the art (e.g. Mayer and Walter, eds. *Immunochemical Methods in Cell and Molecular Biology*, Academic Press, London, 1987).
Monoclonal antibodies, reactive against the polypeptide according to the invention (or variants or fragments thereof) according to the present invention, can be prepared by immunizing inbred mice by techniques also known in the art (Kohler and Milstein, *Nature*, 256, 495-497, 1975).

Methods for large-scale production of antibodies according to the invention are also known in the art. Such methods rely on the cloning of (fragments of) the genetic information encoding the protein according to the invention in a filamentous phage for phage display. Such techniques are described i.a.in review papers by Cortese, R. et al., (1994) in Trends Biotechn. 12: 262-267., by Clackson, T. & Wells, J.A. (1994) in Trends Biotechn. 12: 173-183, by Marks, J.D. et al., (1992) in J. Biol. Chem. 267: 16007-16010, by Winter, G. et al., (1994) in Annu. Rev. Immunol. 12: 433-455, and by Little, M. et al., (1994) Biotechn. Adv. 12: 539-555. The phages are subsequently used to screen camelid expression libraries expressing camelid heavy chain antibodies. (Muyldermans, S. and Lauwereys, M., Journ. Molec. Recogn. 12: 131-140 (1999) and Ghahroudi, M.A. et al., FEBS Letters 414: 512-526 (1997)). Cells from the library that express the desired antibodies can be replicated and subsequently be used for large scale expression of antibodies.

Examples

Example 1:

Isolation of *Lawsonia intracellularis* from infected porcine ilea.

L. intracellularis infected ilea, confirmed by histopathology and acid-fast Ziehl-Neelsen staining, were collected from pigs **that** died with PE, and stored at -80°C. After thawing *L. intracellularis* bacteria were isolated from mucosal scrapings taken from the infected intestinal wall. The ileal scrapings were homogenized repeatedly in PBS in an omnimixer to release the intracellular bacteria as described by Lawson et al. (Vet. Microbiol. 10: 303-323 (1985)). Supernatant obtained after low-speed centrifugation to remove cell debris was filtered through 5.0, 3.0, 1.2, and 0.8 µm filters (Millipore). The filtrate was subsequently centrifuged at 8000 g for 30 min, giving a small pellet of *L. intracellularis* bacteria. These bacteria were further purified using a Percoll gradient. The identity of the purified bacteria was assessed by PCR (Jones et al., J. Clin. Microbiol. 31: 2611-2615 (1993)) whereas purity of the isolated bacteria (>95%) was assessed by phase contrast microscopy to reveal any contaminating bacteria or gut debris present.

Bacterial strains and plasmids

L. intracellularis cells were isolated from infected ileal material as described above. *Escherichia coli* host strain BL21star(DE3) containing vector pLysSrare and plasmid pET-His-1 were purchased from Novagen (Madison, Wisconsin, USA). *E. coli* strain TOP10F' was purchased from Invitrogen (Groningen, the Netherlands). Stocks of all bacterial strains, containing 30% glycerol, were stored at –70°C.
Luria Bertani broth (LB) and LB plates were prepared according to standard procedures.

DNA isolation

In order to obtain highly purified *L. intracellularis* chromosomal DNA, DNA was prepared from bacterial cells using a Biorad chromosomal DNA isolation kit (Biorad, Veenendaal, the Netherlands). Plasmid DNA was isolated using Qiagen products.

PCR amplification

PCR amplification was performed using a PCR mixture containing 52 U/ml Expand High Fidelity Enzyme Mix (Roche Applied BioSciences), Expand HF buffer with 2.5 mM $MgCl_2$, 16 mM dNTPs (Promega, Wisconsin, USA), 20 pmoles of primers and 15 ng chromosomal DNA of *L. intracellularis* as template.
For standard applications (i.e. colony PCR) the PCR mixture contained 20 U/ml Supertaq and Supertaq buffer (HT Biotechnology Ltd, Cambridge, UK), containing 8 mM dNTPs (Promega, Wisconsin, USA), 10 pmoles of primers and 15 ng template.

Ligation and transformation

Ligations were performed in a 1 x ligation buffer with 1 unit of ligation enzyme (Gibco BRL Life Technologies Inc., USA) at 16 °C overnight. 1 µl of the ligation reaction was transformed to *E. coli* competent cells by heat shock. The BL21star(DE3) *E. coli* competent cells and the TOP10F' *E .coli* competent cells were made competent using standard methods.

Expression of 8xHIS fusion proteins

For the 75 kD, 27 kD, 62 kD and the 57 kD gene, the following expression method was used. The DNA sequence of the expression vector was confirmed by standard sequencing techniques before the expression vector was transformed to BL21star(DE3) containing pLysSrare. The resulting strain was grown overnight at 37°C at 200 rpm in 5 ml LB with 100 µg/ml ampicillin. The overnight culture was diluted 1:100 in 50 ml LB with 100 µg/ml ampicillin. This culture was grown under the same conditions until the OD_{600} reached 0.5. The culture was induced with IPTG to a final concentration of 1mM and continued to grow for a subsequent 3 hours. 100 µl samples were taken for analysis. *E. coli* strain BL21star(DE3) containing pLysSrare was grown and induced under the same conditions and samples were taken as a negative control. The samples were analyzed by SDS page.

In vitro transcription and translation

For the 74 kD, 44 kD, 43 kD, 26/31 kD and the 101 kD gene, the following expression method was followed. In vitro transcription and translation was performed

using the Rapid Translation System from Roche Applied Science (Mannheim, FRG) according the manufacturer's protocol.

Summarizing, first the knowledge based sequence-optimization tool ProteoExpert RTS E. coli HY was used to design high yield variants of the original gene. This program optimizes the DNA template for the translation step by suggesting mutations in the DNA sequence. Only silent mutations were allowed, leading to identical amino-acid sequences on the protein level. However, changes of up to 8 nucleotides within the first 6 codons were proposed by the ProteoExpert service to give better expression results.

Ten sense and a universal antisense primers, containing a 5' overlapping region of 20 nucleotides and 30-38 additional gene-specific nucleotides, were used in 10 different PCR reactions to amplify these variants with purified *L. intracellularis* chromosomal DNA as template. The obtained amplicons were purified from gel and used for the generation of linear expression constructs for cell-free protein expression using the RTS E. coli Linear Template Generation Set, His-tag, to introduce the necessary T7 regulatory elements.

Again the obtained amplicons were purified from gel, and after quantification, the appropriate amount of DNA was used for protein expression analysis in a 50-µl RTS 100 E. coli HY reaction mixture. Expression was analysed using Western blotting with an anti polyhistidine monoclonal antibody.

The construct that gave the highest protein yields was ligated to pCR2.1 TOPO TA vector using the TOPO TA cloning kit. The obtained plasmid was used for medium scale protein production using the RTS 500 E. coli HY kit. The samples were analyzed by SDS page and by Western blot.

The DNA sequence of the expression vector was confirmed using an ABI 310 automated sequencer (Applied Biosystems, California, USA).

If needed protein was purified using TALON immobilized metal affinity chromatography resin according to the protocol of the manufacturer for purification using denaturing conditions. Subsequently, the purified protein fraction was dialyzed against PBS to remove urea from the sample.

Polyacrylamide gel electrophoresis and western blotting

SDS-PAGE was performed using 4-12% Bis-Tris gels from the NuPAGE electrophoresis system (Invitrogen, www.invitrogen.com). Western blotting was performed using semi dry blotting procedures. Western blots were developed using chicken anti-Lawsonia polyclonal serum that was raised against a whole cell preparation in a water:oil=45:55 emulsion or using a pig serum that had been obtained from a animal that been orally challenged with purified *L. intracellularis* cells and that had developed clinical signs and post-mortem lesions typical for *L. intracellularis* infection. The sera were pre-adsorbed using an equal volume crude cell extracts from BL21star(DE3) containing vector pLysSrare at 4°C for 4 hours.

Results

Cloning of *L. intracellularis* gene 5074 in T7 based expression vector

Gene 5074 was amplified using primer 2075 (CATGCCATGGCTAGTCTTACAGCAGGAATGTG) [SEQ ID NO.: 19] and 2076 (CCGCTCGAGACACGCTTCATATTTACAACTG) [SEQ ID NO.: 20] . In the process a 5` NcoI and 3` XhoI site were introduced into the PCR product. The obtained PCR product was digested using restriction enzymes NcoI and XhoI. The digested PCR product was subsequently ligated to pET22b that had been cut with the same two restriction enzymes. The ligation mixture was transformed to *E. coli* TOP10F and incubated o/n at 37°C. Putative transformants were checked for the right plasmid, using colony PCR. The plasmid inserts, of colony PCR positive transformants, were checked by nucleotide sequence analysis. One of the clones that contained a sequence as expected on basis of the cloning strategy was chosen and designated pET5074.

Expression of *L. intracellularis* gene 5074 from T7 promoter in *E. coli*

Plasmid pET5074 was transformed to BL21Star(DE3)pLysSrare. The resulting strain was tested for recombinant protein production as described above. Samples of the induced culture and control samples were analysed by SDS-PAGE gel electrophoresis (Fig. 1A). A clear protein band of approximately 75 kD was observed in sample that had been taken after 3 hours of induction (Fig. 1A, lane 3) in comparison with the un-induced sample (Fig. 1A, lane 2).

The same samples were also analysed by western blot using the pig and chicken serum. A reaction with the 75 kD protein was observed using the serum from the pig that had been orally challenged with purified *L. intracellularis* cells (Fig 1B, lane 3). and with the chicken anti-*L. intracellularis* serum (Fig 1C, lane 3).

Conclusion: the 75 kD vaccine component could be successfully expressed in large quantities and is indeed clearly recognized by both orally challenged pig anti-*L. intracellularis* serum and by chicken anti-*L. intracellularis* serum

Cloning of *L. intracellularis* gene 5669 in T7 based expression vector

Gene 5669 was amplified using primer 2185 (CATGCCATGGATGCACTTGAGTTCATACAAGA) [SEQ ID NO.: 21] and 2186 (CCGCTCGAGATGAATTTGGATTTCAATTT) [SEQ ID NO.: 22]. In the process a 5` NcoI and 3` XhoI site were introduced into the PCR product. The obtained PCR product was digested using restriction enzymes NcoI and XhoI. The digested PCR product was subsequently ligated to pET22b that had been cut with the same two restriction enzymes. The ligation mixture was transformed to *E. coli* TOP10F and incubated o/n at 37°C. Putative transformants were checked for the right plasmid, using colony PCR. The plasmid inserts, of colony PCR positive transformants, were checked by nucleotide sequence analysis. One of the clones that contained a sequence as expected on basis of the cloning strategy was chosen and designated pET5669.

Expression of *L. intracellularis* gene 5669 from T7 promoter in *E. coli*

Plasmid pET5669 was transformed to BL21Star(DE3)pLysSrare. The resulting strain was tested for recombinant protein production as described above. Samples of the induced culture and control samples were analysed by SDS-PAGE gel electrophoresis (Fig. 2A). A clear protein band of approximately 27 kDa was observed in sample that had been taken after 3 hours of induction (Fig. 2A, lane 3) in comparison with the uninduced sample (Fig. 2A, lane 2).

The same samples were also analysed by western blot using the pig and chicken serum. A reaction with the 27 kD protein was observed using the serum from the pig

that had been orally challenged with purified *L. intracellularis* cells (Fig 2B, lane 3). and with the chicken anti-*L. intracellularis* serum (Fig 2C, lane 3).

Conclusion: the 27 kD vaccine component could be successfully expressed in large quantities and is indeed clearly recognized by both orally challenged pig anti-*L. intracellularis* serum and by chicken anti-*L. intracellularis* serum

Cloning of *L. intracellularis* gene 4423 in T7 based expression vector

Gene 4423 was amplified using primer 2171 (CATGCCATGGATGCTAGCTATGTGGTTTTGCC) [SEQ ID NO.: 23] and 2172 (CCGCTCGAGGTTATCTTCAACAGCCTTAG) [SEQ ID NO.: 24]. In the process a 5` NcoI and 3` XhoI site were introduced into the PCR product. The obtained PCR product was digested using restriction enzymes NcoI and XhoI. The digested PCR product was subsequently ligated to pET22b that had been cut with the same two restriction enzymes. The ligation mixture was transformed to *E. coli* TOP10F and incubated o/n at 37°C. Putative transformants were checked for the right plasmid, using colony PCR. The plasmid inserts, of colony PCR positive transformants, were checked by nucleotide sequence analysis. One of the clones that contained a sequence as expected on basis of the cloning strategy was chosen and designated pET4423.

Expression of *L. intracellularis* gene 4423 from T7 promoter in *E. coli*

Plasmid pET4423 was transformed to BL21Star(DE3)pLysSrare. The resulting strain was tested for recombinant protein production as described above. Samples of the induced culture and control samples were analysed by SDS-PAGE gel electrophoresis (Fig. 3A). A clear protein band of approximately 62 kDa was observed in **a** sample that had been taken after 3 hours of induction (Fig. 3A, lane 3) in comparison with the uninduced sample (Fig. 3A, lane 2).

The same samples were also analysed by western blot using the pig serum. A reaction with the 62 kD protein was observed using the serum from the pig that had been orally challenged with purified *L. intracellularis* cells (Fig 3B, lane 3).

Conclusion: the 62 kD vaccine component could be successfully expressed in large quantities and is indeed clearly recognized by orally challenged pig anti-*L. intracellularis* serum.

Cloning of *L. intracellularis* gene 3123 in T7 based expression vector

Gene 3123 was amplified using primer 2167 (CATGCCATGGATCAGTTTAATAAACCCTCTTT) [SEQ ID NO.: 25] and 2168 (CCGCTCGAGGGTTCGACCATGTACAAACT) [SEQ ID NO.: 26]. In the process a 5` NcoI and 3` XhoI site were introduced into the PCR product. The obtained PCR product was digested using restriction enzymes NcoI and XhoI. The digested PCR product was subsequently ligated to pET22b that had been cut with the same two restriction enzymes. The ligation mixture was transformed to *E. coli* TOP10F and incubated o/n at 37°C. Putative transformants were checked for the right plasmid, using colony PCR. The plasmid inserts, of colony PCR positive transformants, were checked by nucleotide sequence analysis. One of the clones that contained a sequence as expected on basis of the cloning strategy was chosen and designated pET3123.

Expression of *L. intracellularis* gene 3123 from T7 promoter in *E. coli*

Plasmid pET3123 was transformed to BL21Star(DE3)pLysSrare. The resulting strain was tested for recombinant protein production as described above. Samples of the induced culture and control samples were analysed by SDS-PAGE gel electrophoresis (Fig. 4A). A protein band of approximately 57 kDa was observed in sample that had been taken after 3 hours of induction (Fig. 4A, lane 3) in comparison with the uninduced sample (Fig. 4A, lane 2).

The same samples were also analysed by western blot using the pig serum. A reaction with the 57 kD protein was observed using the serum from the pig that had been orally challenged with purified *L. intracellularis* cells (Fig 4B, lane 3).

Conclusion: the 57 kD vaccine component could be successfully expressed in large quantities and is indeed clearly recognized by whole-cell vaccinated pig anti-*L. intracellularis* serum.

Cloning of *L. intracellularis* gene 5293

For the evaluation of the ProteoExpert suggestions, linear DNA templates were generated via PCR using the RTS Linear Template Generation Set. The primers used in these experiments also introduced a His6-tag at the C-terminus for detection and purification. The PCR-generated templates were examined for their expression performance using RTS 100 E. coli HY Kit. The suggested DNA sequence that gave the highest yields was constructed using primers 5293A5 and 5293B (Table 1) in the first PCR.

The obtained expression construct was ligated to pCR2.1 TOPO TA vector and the resulting vector was transformed to *E. coli* TOP10F and incubated o/n at 37°C. Putative transformants were checked for the right plasmid, using colony PCR. The plasmid inserts, of colony PCR positive transformants, were checked by nucleotide sequence analysis. One of the clones that contained a sequence as expected on basis of the cloning strategy was chosen and designated pTOPO5293.

Table 1. Sequence of the degenerated primers used for the amplification of gene 5293.

Primer	Sequence
5293A5	CTTTAAGAAGGAGATATACCATGGCGGATTATTTAA GTGGTGGAATTTCTTTTGGAGG [SEQ ID NO.: 27]
5293B	TGATGATGAGAACCCCCCCCTGCACCAAGTTGCC [SEQ ID NO.: 28]

Expression of *L. intracellularis* gene 5293 using RTS technology

Plasmid pTOPO5293 was purified from *E. coli* TOP10F and the appropriate amount of DNA was added to a RTS500 vial. After incubation conform the protocol of the manufacturer, a sample was taken for analysis using SDS-PAGE gel electrophoresis (Fig. 5A). A clear protein band of approximately 74 kDa was observed in sample that had been taken after 30 hours of induction (Fig. 5A, lane 3) in comparison with the control sample (Fig. 5A, lane 2).

The same samples were also analysed by western blot using pig serum. The 74 kD protein was specifically recognized by the polyclonal pig serum used in this experiment (Fig 5B, lane 3).

Conclusion: The 74 kD protein according to the invention can efficiently be expressed and is specifically recognized by the polyclonal pig serum. The 74 kD protein is an important vaccine component for the protection of pigs against *Lawsonia intracellularis* infection.

Cloning of *L. intracellularis* gene 5464

For the evaluation of the ProteoExpert suggestions, linear DNA templates were generated via PCR using the RTS Linear Template Generation Set. The primers used in these experiments also introduced a His_6-tag at the C-terminus for detection and purification. The PCR-generated templates were examined for their expression performance using RTS 100 *E. coli* HY Kit. The suggested DNA sequence that gave the highest yields was constructed using primers 5464A5 and 5464B (Table 2) in the first PCR.

The obtained expression construct was ligated to pCR2.1 TOPO TA vector and the resulting vector was transformed to *E. coli* TOP10F and incubated o/n at 37°C. Putative transformants were checked for the right plasmid, using colony PCR. The plasmid inserts, of colony PCR positive transformants, were checked by nucleotide sequence analysis. One of the clones that contained a sequence as expected on basis of the cloning strategy was chosen and designated pTOPO5464.

Table 2. Sequence of the degenerated primers used for the amplification of gene 5464.

Primer	Sequence
5464A5	CTTTAAGAAGGAGATATACCATGGCTAACG TATCAGGAATTCCTGCACCACGATT [SEQ ID NO.: 29]
5464B	TGATGATGAGAACCCCCCCCTTGTATATTATTTTCATCTG [SEQ ID NO.: 30]

Expression of *L. intracellularis* gene 5464 using RTS technology

Plasmid pTOPO5464 was purified from *E. coli* TOP10F and the appropriate amount of DNA was added to an RTS500 vial. After incubation according to the protocol of

the manufacturer, (Roche Diagnostics GmbH, Mannheim, Germany), a sample was taken for analysis using SDS-PAGE gel electrophoresis (Fig. 6A). A clear protein band of approximately 44 kDa was observed in sample that had been taken after 30 hours of induction (Fig. 6A, lane 3) in comparison with the control sample (Fig. 6A, lane 2). Using the anti-polyhistidine monoclonal in Western blot revealed a second reactive protein suggesting the presence of an internal translation start site in the gene or post translational modification of the mature protein (Fig 6B, lane 3).
A polyclonal serum was raised against purified 44 kD protein. In ELISA this serum specifically recognized purified whole *L. intracellularis* cells that were used as coating material with a reasonable titer (>2log9). Low titers were measured using a control serum (<2log3).

Conclusion: The 44 kD protein according to the invention can efficiently be expressed. Moreover, antiserum raised against the expressed protein is perfectly capable of recognizing *Lawsonia intracellularis* cells. The 44 kD protein is an important vaccine component for the protection of pigs against *Lawsonia intracellularis* infection.

Cloning of *L. intracellularis* gene 5473

For the evaluation of the ProteoExpert suggestions, linear DNA templates were generated via PCR using the RTS Linear Template Generation Set. The primers used in these experiments also introduced a His6-tag at the C-terminus for detection and purification. The PCR-generated templates were examined for their expression performance using RTS 100 E. coli HY Kit. The suggested DNA sequence that gave the highest yields was constructed using primers 5473A2 and 5473B (Table 3) in the first PCR.
The obtained expression construct was ligated to pCR2.1 TOPO TA vector and the resulting vector was transformed to *E. coli* TOP10F and incubated o/n at 37°C. Putative transformants were checked for the right plasmid, using colony PCR. The plasmid inserts, of colony PCR positive transformants, were checked by nucleotide

sequence analysis. One of the clones that contained a sequence as expected on basis of the cloning strategy was chosen and designated pTOPO5473.

Table 3. Sequence of the degenerated primers used for the amplification of gene 5473.

Primer	Sequence
5473A2	CTTTAAGAAGGAGATATACCATG ACAAATTTTGGAGATATTAGCGGAAGCTCCG [SEQ ID NO.: 31]
5473B	TGATGATGAGAACCCCCCCCCTCACGTGCACCA [SEQ ID NO.: 32]

Expression of *L. intracellularis* gene 5473 using RTS technology

Plasmid pTOPO5473 was purified from *E. coli* TOP10F and the appropriate amount of DNA was added to a RTS500 vial. After incubation according to the protocol of the manufacturer, (Roche Diagnostics GmbH, Mannheim, Germany), a sample was taken for analysis using SDS-PAGE gel electrophoresis (Fig. 7A). However, it was impossible to see whether the reaction mixture had produced a protein of around 40 kDa because the mixture already contains a dominant protein of around 40 kDa (Fig. 7A, lane 2 and 4). The RTS 500 reaction containing pTOPO5473 and the control mixture were loaded onto a IMAC column and proteins that had bound to the column were analyzed using SDS-PAGE. From the gel it appeared that a 43 kDa protein was eluted from the column (Fig 7A, lane 5) that was not purified from the control sample (Fig. 7A, lane 3), so protein was expressed.

The same samples were also analysed by Western blot using pig-derived and chicken-derived *L. intracellularis* anti-serum. A reaction with the 43 kD protein was observed both using serum from *L. intracellularis* bacterin vaccinated pigs (Fig 7B, lane 5) and chickens (Fig 7C, lane 5).

Conclusion: The 43 kD protein according to the invention can be efficiently expressed. Moreover, antiserum raised against *Lawsonia intracellularis* cells from both chickens and pigs recognizes the expressed protein. The 43 kD protein is an important vaccine component for the protection of pigs against *Lawsonia intracellularis* infection.

Cloning of *L. intracellularis* gene 4320

For the evaluation of the ProteoExpert suggestions, linear DNA templates were generated via PCR using the RTS Linear Template Generation Set. The primers used in these experiments also introduced a His_6-tag at the C-terminus for detection and purification. The PCR-generated templates were examined for their expression performance using RTS 100 *E. coli* HY Kit. The suggested DNA sequence that gave the highest yields was constructed using primers 4320A8 and 4320B (Table 4) in the first PCR.

The obtained expression construct was ligated to pCR2.1 TOPO TA vector and the resulting vector was transformed to *E. coli* TOP10F and incubated o/n at 37°C. Putative transformants were checked for the right plasmid, using colony PCR. The plasmid inserts, of colony PCR positive transformants, were checked by nucleotide sequence analysis. One of the clones that contained a sequence as expected on basis of the cloning strategy was chosen and designated pTOPO4320.

Table 4. Sequence of the degenerated primers used for the amplification of gene 4320.

Primer	Sequence
4320A8	CTTTAAGAAGGAGATATACC ATGAGCTTAGTAATTAACAATAACCTGATGGCCG [SEQ ID NO.: 33]
4320B	TGATGATGAGAACCCCCCCCGCCAATAAGTTGCTG [SEQ ID NO.: 34]

Expression of *L. intracellularis* gene 4320 using RTS technology

Plasmid pTOPO4320 was purified from *E. coli* TOP10F and the appropriate amount of DNA was added to an RTS500 vial. After incubation according to the protocol of the manufacturer, (Roche Diagnostics GmbH, Mannheim, Germany), a sample was taken for analysis using SDS-PAGE gel electrophoresis (Fig. 8A). Two clear protein bands of approximately 31 and 26 kDa were observed in sample that had been taken after 30 hours of induction (Fig. 8A, lane 3) in comparison with the control sample (Fig. 8A, lane 2). Both bands reacted with an anti-polyhistidine monoclonal

suggesting the presence of an internal translation start site in the gene or post translational modification of the mature protein (Fig 8B, lane 2). Using polyclonal pig and chicken sera, high titers (>2log10) were observed in an ELISA using purified 26/31 kD protein and purified whole *L. intracellularis* cells as coating material. Using IHC we found that polyclonal anti-26/31 kD protein specifically recognized *L. intracellularis* infected enterocytes, whereas no reaction was seen in with specimens cut from the ilia of healthy pigs. The serum used in IHC also gave high titers (>2log15) against whole *L. intracellularis* cells in ELISA.

Conclusion: The 26/31 kD protein according to the invention can be efficiently expressed. Moreover, antiserum raised against *Lawsonia intracellularis* cells from both chickens and pigs recognizes the expressed protein in ELISA tests, where the wells were coated with the 26/31 kD protein.
Moreover, polyclonal anti-serum raised against the 26/31 kD protein specifically recognized *L. intracellularis* infected enterocytes.
The 26/31 kD protein is an important vaccine component for the protection of pigs against *Lawsonia intracellularis* infection.

Cloning of *L. intracellularis* gene 2008

Sequence analysis of gene 2008 had revealed that the gene encoded a putative N-terminal signal sequence and a C-terminal beta-barrel structure. Both structures are known to be very hydrophobic. Because the RTS system has been found unsuitable for the expression of proteins that contain large hydrophobic regions it was decided to amplify gene 2008 from base 37 to 1958. Expression of this gene fragment resulted in a protein of 63 kD.
For the evaluation of the ProteoExpert suggestions, linear DNA templates were generated via PCR using the RTS Linear Template Generation Set. The primers used in these experiments also introduced a His_6-tag at the C-terminus for detection and purification. The PCR-generated templates were examined for their expression performance using RTS 100 E. coli HY Kit. The suggested DNA sequence that gave

the highest yields was constructed using primers 2008A6 and 2008B (Table 5) in the first PCR.

The linear expression construct was ligated to pCR2.1 TOPO TA vector and the resulting vector was transformed to *E. coli* TOP10F and incubated o/n at 37°C. Putative transformants were checked for the right plasmid, using colony PCR. The plasmid inserts, of colony PCR positive transformants, were checked by nucleotide sequence analysis. One of the clones that contained a sequence as expected on basis of the cloning strategy was chosen and designated pTOPO2008.

Table 5. Sequence of the degenerated primers used for the amplification of gene 2008.

Primer	Sequence
2008A6	CTTTAAGAAGGAGATATACCATGGCAGATGTAT TTTTCGAAGGCAGAACCGAAAC [SEQ ID NO.: 35]
2008B	TGATGATGAGAACCCCCCCCATTAACATACCAAATAGAT [SEQ ID NO.: 36]

Expression of *L. intracellularis* gene 2008 using RTS technology

Plasmid pTOPO2008 was purified from *E. coli* TOP10F and the appropriate amount of DNA was added to an RTS500 vial. After incubation according to the protocol of the manufacturer, (Roche Diagnostics GmbH, Mannheim, Germany), a sample was taken for analysis using SDS-PAGE gel electrophoresis (Fig. 9A). A clear protein band of approximately 63 kDa was observed in sample that had been taken after 30 hours of induction (Fig. 9A, lane 3) in comparison with the control sample (Fig. 9A, lane 2).

The same samples were also analysed by western blot using both pig- and chicken-antiserum. A strong reaction with the 63 kD protein was observed using both the polyclonal pig (Fig 9B, lane 3) and chicken serum (Fig 9C, lane 3).

Concusion: the 63 kD fragment of the protein according to the invention can efficiently be expressed. Moreover, the 63 kD protein fragment is strongly and equally well recognized by both chicken- and pig-antiserum against *Lawsonia intracellularis* cells.

The 101 kD protein according to the invention and the 63 kD protein fragment thereof are important vaccine components for the protection of pigs against *Lawsonia intracellularis* infection.

Example 2:

The objective of this experiment was to test for active protection in pigs induced by experimental *Lawsonia* recombinant combi subunit vaccine comprising the 75 kD, 44 kD, 26/31 kD and the 27 kD protein.

Vaccine

Inactivated recombinant subunit combi vaccine in micro-Diluvac Forte.
The following antigens were incorporated: 75 kD, 44 kD, 26/31 kD and the 27 kD protein. The mixture of recombinant antigens was dialyzed against a dialysis buffer (50 mM Tris-HCl pH8.0, 100 mM NaCl, 1 mM EDTA, 1mM oxidized glutathione, 3 mM reduced glutathione, 10 mM CHAPS) and concentrated using PEG20,000. The concentration of all antigens was estimated from Coomassie stained NuPage gel using Gene Tools software (Syngene, Cambridge, England). The antigens were formulated in the vaccine at a concentration of 50 µg of every single antigen per ml.

Experimental design

Eighteen 6-week-old SPF pigs (3 groups of 6 pigs each) were used for the experiment. Group 1 pigs were vaccinated intramuscularly (neck) with 2 ml of the recombinant combi subunit vaccine at T=0 and T=4w. Group 2 was left as non-vaccinated challenge control group. Group 3 was a non-treated performance control group.
At T=6w (12 weeks of age) groups 1 and 2 were challenged orally with homogenized infected mucosa. Subsequently all pigs were daily observed for clinical signs of Porcine Proliferative Enteropathy (PPE) during 3 weeks.
At T=9w (15-week-old) all remaining pigs were euthanized and post-mortem examined. The intestines (ileum) were examined for macroscopical changes typical

for *Lawsonia intracellularis* infection and samples were taken for histological examination.

Preparation of challenge material

Challenge material was prepared by scraping the mucosa of the ileum of confirmed PPE cases. The material was stored in batches of 200 grams at -20^{0}C ntil further use. Shortly before challenge, 200 gram of the infected mucosa was thawed and mixed with 200 ml physiological salt solution. This mixture was homogenized in an omnimixer for one minute at full speed and then further diluted with 400 ml physiological salt solution (up to 800 ml).

Vaccination

The pigs were assigned to 3 treatment groups as described below.

Group	Number	Unit	Vaccine	Dose	Route	Time in weeks
1	6	5	Sub-unit vaccine	2 ml	IM	T=0 and T=4
2	6	5	Non-vaccinated Challenge control	-	-	-
3	6	12	Non-vaccinated Negative control	-	-	-

Challenge

Group 1 and 2 were challenged orally with 20 ml challenge inoculum at T=6w (12 weeks of age). Group 3 was left as a non-treated control group.

Post-mortem examination and histopathology

All pigs were killed at T=9w (15 weeks of age, 3 weeks after challenge) and subjected to a post-mortem examination to assess the efficacy of the different vaccines.

-	Normal
1	minimal to mild redness/erosions without thickening of mucosa over limited area
2	mild to moderate redness/erosions and/or thickening of mucosa

	over limited area
3	moderate redness/erosions and/or thickening of mucosa over extended area
4	moderate to severe redness/erosions and/or ulceration and/or severe thickening of mucosa and intestinal wall over extended area

From each pig at least one sample of the ileum (if present from an affected area) was taken for histology.

Histologic scoring of ileum samples was based on:

a) Presence of *L. intracellularis* bacteria in slides: Warthin Starry was performed for detection of bacteria.

b) Evaluation of histologic lesions in Hematoxylin/Eosin slides: Severity of *L. intracellularis*-specific lesion (adenomatous glandular proliferation) was scored. Other lesions are described.

Histologic lesion		Warthin Starry	HE lesion score	Remark
No abnormalities detected		0	0	
Adenomatous glandular proliferation	mild (multi)focal	2	1	Typical PPE
	moderate diffuse or multifocal	2	2	
	severe diffuse	2	3	
Other lesions (not going along with adenomatous proliferation)		0/1	Lesion is described	

Warthin Starry:

0: no bacteria evident

1: presence of single/small numbers of bacteria within lesion

2: presence of considerable numbers of bacteria within lesion

Evaluation of results

All data were recorded for each pig individually. The mean score per group was calculated for the parameters histopathology score and post-mortem score. Starting from the score of the challenge control group, the % protection in the vaccinated was calculated. Pathology scores were compared using two-sided Mann-Whitney U test.

Results

Post-mortem and histology

Post-mortem results after challenge are shown in Table 6. Histopathological scores showed clear cut results. Only the control pigs showed the typical histopathological lesions (=severe diffuse adenomatous glandular proliferation) and only the enterocytes in the control histopathology slides contained numerous *Lawsonia* bacteria, whereas no bacteria were observed in the enterocytes of the vaccinated and non-challenged animals.

Conclusion

The post-mortem and histopathological examination gave clear cut results. The subunit vaccine tested appeared to induce 100% protection against histopathological lesions and the occurrence of *Lawsonia* in the enterocytes (=against infection).

Table 6 Post-mortem examination 3 weeks after challenge (T=9w)

Table 1 Post-mortem examination 3 weeks after challenge (T=9w)

group	pig #	Histo-pathological scores Macroscopically ileum	Histological WS	HE	remarks / description
1 sububit in μ-DF IM route 2x	177	3	0	0	NAD
	180	2	0	0	NAD
	356	3	0	0	one crypt abscess, one focus of mild GP
	361	0	0	0	NAD
	378	2	0	0	mild focal GP, thick tunica muscularis
	385	2	0	0	mild focal GP, thick tunica muscularis
	total	12	0	0	
	p-value[a]	**0.054**		**0.000**	
2 challenge control	181	4	2	3	severe diffuse adematous GP
	193	3	2	2	moderate diffuse adematous GP
	194	3	2	3	severe diffuse adematous GP
	195	4	2	3	severe diffuse adematous GP
	351	2	NS	NS	NS
	376	4	2	3	severe diffuse adematous GP
	total	20	10	14	
3 untreated contact controls	173	2	0	0	NAD
	174	2	0	0	NAD
	179	0	0	0	NAD
	183	1	0	0	NAD
	200	1	0	0	NAD
	388	0	0	0	NAD
	total	6	0	0	
	p-value[a]	**0.008**		**0.000**	

[a] two-sided Mann-Whitney U test (compared to control group 3)
WS=Warthin Starry staining, HE=haematoxilin-eosin staining, NAD=no abnormality detected, NS=no sample
GP=glandular proliferation, MFPC=multifocal propria congestion
N.B. all group 4 pigs showed (macroscopically) congestion of lymph vessels

Legend to the figure.

Fig. 1. Analysis of the over-expression of *Lawsonia intracellularis* gene 5074 in *Escherichia coli* BL21STAR/pLysSRARE by SDS-PAGE (A) and Western blotting with polyclonal pig serum (B) and polyclonal chicken serum (C). Lane 1, molecular weight marker; lane 2, pET5074 T=0; lane 3, pET5074 T=3. Arrows indicate the location of the expression product.

Fig. 2. Analysis of the over-expression of *Lawsonia intracellularis* gene 5669 in *Escherichia coli* BL21STAR/pLysSRARE by SDS-PAGE (A) and Western blotting with polyclonal pig serum (B) and polyclonal chicken serum (C). Lane 1, molecular weight marker; lane 2, pET5669 T=0; lane 3, pET5669 T=3. Arrows indicate the location of the expression product.

Fig. 3. Analysis of the over-expression of *Lawsonia intracellularis* gene 4423 in *Escherichia coli* BL21STAR/pLysSRARE by SDS-PAGE (A) and Western blotting with polyclonal pig serum (B). Lane 1, molecular weight marker; lane 2, pET4423 T=0; lane 3, pET4423 T=3. Arrows indicate the location of the expression product.

Fig. 4. Analysis of the over-expression of *Lawsonia intracellularis* gene 3123 in *Escherichia coli* BL21STAR/pLysSRARE by SDS-PAGE (A) and Western blotting with polyclonal pig serum (B). Lane 1, molecular weight marker; lane 2, pET3123 T=0; lane 3, pET3123 T=3. Arrows indicate the location of the expression product.

Fig. 5. Analysis of the expression of *Lawsonia intracellularis* gene 5293 using RTS500 technology by SDS-PAGE (A) and Western blotting with polyclonal pig serum (B). Lane 1, molecular weight marker; lane 2, control; lane 3, pET5293 Arrows indicate the location of the expression product.

Fig. 6. Analysis of the expression of *Lawsonia intracellularis* gene 5464 using RTS500 technology by SDS-PAGE (A) and Western blotting using anti-polyhistidine monoclonal (B). Lane 1, molecular weight marker; lane 2, control; lane 3, pET5464

Arrows indicate the location of the expression product.

Fig. 7. Analysis of the expression of *Lawsonia intracellularis* gene 5473 using RTS500 technology by SDS-PAGE (A) and Western blotting with polyclonal pig serum (B) and polyclonal chicken serum(C). Lane 1, molecular weight marker; lane 2, control; lane 3, bound protein fraction IMAC purification controle sample; lane 4 pET5473; lane 5, bound protein fraction IMAC purification pET5473.
Arrows indicate the location of the expression product.

Fig. 8. Analysis of the expression of *Lawsonia intracellularis* gene 4320 using RTS500 technology by SDS-PAGE (A) and Western blotting using anti-polyhistidine monoclonal (B). Lane 1, molecular weight marker; lane 2, control; lane 3, pET4320
Arrows indicate the location of the expression product.

Fig. 9. Analysis of the expression of *Lawsonia intracellularis* gene 2008 using RTS500 technology by SDS-PAGE (A) and Western blotting with polyclonal pig serum (B). Lane 1, molecular weight marker; lane 2, control; lane 3, pET2008
Arrows indicate the location of the expression product.